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Original research paper

Primaquine-NSAID twin drugs: Synthesis, radical scavenging, antioxidant and Fe²⁺ chelating activity

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Novel primaquine conjugates with non-steroidal anti-inflammatory drugs (PQ-NSAIDs, **4a-h**) were prepared, fully chemically characterized and screened for radical scavenging and antioxidant activities. The synthetic procedure leading to twin drugs **4a-h** involved two steps: *i*) preparation of NSAID benzotriazolides **3a-h** from the corresponding NSAID (ibuprofen, ketoprofen, fenoprofen, ketoprofen hydroxy and methylene analogues, diclofenac or indomethacin) and benzotriazole carboxylic acid chloride (BtCOCl, **1**), *ii*) reaction of intermediates **3a-h** with PQ. The prepared PQ-NSAIDs exerted moderate activities in the DPPH free radical test and β -carotene-linoleic acid assay. Moreover, ketoprofen derivatives **4d** and **4b** demonstrated a notable Fe²⁺ chelating ability as well. On the other hand, negligible antiproliferative and antituberculous effects of conjugates **4a-h** were observed.

Keywords: primaquine, NSAID, twin drug, conjugate, radical scavenging, antioxidant activity, chelating ability

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Excessive production of reactive oxygen species (ROS), associated with inflammation, leads to the condition of oxidative stress. Oxidative stress is a major contributing factor to the high mortality rates associated with several diseases, including malaria, a widespread parasitic disease in the tropical parts of the world. It seems that oxidative stress in malaria plays a dual role. Some authors suggest that oxidative stress seems to contribute to host organism defenses (1). Namely, immune cells use ROS in order to support their functions and therefore need adequate levels of antioxidant defenses in order to avoid the harmful effect of an excessive production of ROS (2). On the other hand, some authors point to deleterious effects of oxidative stress in malaria (3). In addition, some antimalarial agents have oxidative stress-inducing effects that might contribute to the development of side effects such as methemoglobinemia, hemolysis and liver damage (4). Primaquine (PQ) is the only available drug that is active against both the latent liver forms of relapsing malaria caused by *Plasmodium vivax* and *P. ovale* and the gameto-

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cytes from all species of the parasites (5). Modifications of the primary amino group protect PQ against metabolic degradation and lead to an increase in the antimalarial activity (6). In our previous paper (7) we have shown that primaquine urea derivatives possess significant antiradical and antioxidant activities. On the other hand, non-steroidal anti-inflammatory drugs (NSAIDs) and their derivatives have also exerted antioxidant activities (8). We therefore found it worth preparing a series of PQ-NSAID conjugates, twin drugs that combine both drugs in a single drug. In this study, novel primaquine conjugates with ibuprofen, ketoprofen, fenoprofen, diclofenac and indomethacin (PQ-NSAIDs, **4a-h**) were prepared, fully chemically characterized and screened for radical scavenging and antioxidant activity.

A considerable number of iron(III) chelators, as well as certain iron(II) chelators, designed for purposes other than treating malaria have antimalarial activity *in vitro* (9). These facts directed us to check a potential of PQ-NSAIDs conjugates as iron chelators.

A series of urea and carbamate PQ derivatives previously synthesized by our group showed antiproliferative activity (7). On the other hand, NSAIDs are potential anticancer drugs and effective chemopreventive agents (see, for example, ref. 10). Therefore, screening of cytostatic activity on a series of tumour cell lines of PQ-NSAID conjugates was performed as well.

EXPERIMENTAL

Melting points were determined on a Stuart Melting Point Apparatus SMP3 (Barworld Scientific, UK) and were uncorrected. IR spectra were recorded on a FTIR Perkin Elmer Paragon 500 spectrometer (Perkin Elmer, USA). ¹H and ¹³C NMR spectra were recorded on a Varian Gemini 300 spectrometer (Varian, USA), operating at 300 and 75.5 MHz for the ¹H and ¹³C nuclei, respectively. Samples were measured in DMSO-*d*₆ solutions at 20 °C in 5-mm NMR tubes. Chemical shifts (δ) in ppm were referred to TMS. Coupling constants (*J*) are given in Hz. A Perkin Elmer Lambda 25 spectrophotometer (Perkin Elmer, USA) and Stat Fax 3200 (Awareness Technologies, USA) were used for absorbance measurements. Elemental composition of the compounds agreed to within ±0.4 % (CHN-LECO-932, LECO Corporation, USA). For thin-layer chromatography, pre-coated Merck silica gel 60 F254 plates (Merck, Germany) and solvent systems cyclohexane/ethyl acetate/methanol (3:1:0.5) and dichloromethane/methanol (9:1) were used. Spots were visualized by short-wave UV light and iodine vapour. Column chromatography was performed on silica gel of 0.063–0.200 mm (Merck), with cyclohexane/ethyl acetate/methanol (3:1:0.5) or dichloromethane/methanol (9:1) as eluents. A gradual increase of eluent polarity was also applied: first eluent was lipophylic (dichloromethane), while the second (dichloromethane/methanol 99.5:0.5) and the third one (dichloromethane/methanol 99:1) were more polar. Benzotriazole, triphosgene, triethylamine, ethylenediamine, primaquine diphosphate, butylated hydroxyanisole (BHA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, Folin-Ciocalteu reagent, linoleic acid, Tween-40 (polyoxyethylene sorbitan monopalmitate), quercetin and 10 % Pd/C were purchased from Sigma-Aldrich (USA). NSAIDs (diclofenac, ketoprofen, fenoprofen, ibuprofen and indomethacin) were obtained as gift samples from Pliva and Belupo (Croatia) and the

University of Potchefstroom (Republic of South Africa). Primaquine diphosphate and NSAIDs were used as racemates. Other chemicals and solvents used were of analytical grade. 1-Benzotriazole carboxylic acid chloride (**1**), 2-(3-benzylphenyl)propanoic acid (**2a**), 2-(3-(hydroxy(phenyl)methyl)phenyl)propanoic acid (**2b**) and NSAID benzotriazolides **3a–h** were prepared according to our published procedures (11, 12). Primaquine base was prepared from primaquine diphosphate prior to use. Primaquine solution was protected against light during the whole procedure.

Synthesis of PQ-NSAID twin drugs (4a–h). General procedure

A light-protected solution of primaquine (0.156 g, 0.6 mmol), appropriate benzotriazolidine **3** (0.5 mmol) and triethylamine (TEA) (0.209 mL, 1.5 mmol) in toluene (5 mL) was stirred at room temperature for 0.5 h. Synthesis of **4h** was performed with a double amount of primaquine and TEA, while for synthesis of **4g** only half the amount of TEA was used. The reaction mixture was extracted with diluted NaOH solution pH 9 (5 × 10 mL). The organic layer was washed with water, dried over anhydrous sodium sulphate, filtrated and evaporated to yield a crude product.

N-(4-(6-methoxyquinolin-8-ylamino)pentyl)-2-(4-isobutylphenyl)propanamide (**4a**). – Reactant: 1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-(4-isobutylphenyl)propan-1-one (benzotriazolidine **3a**) (0.154 g, 0.5 mmol). The crude product was purified by column chromatography (eluent: dichloromethane/methanol 9:1).

N-(4-(6-methoxyquinolin-8-ylamino)pentyl)-2-(3-benzylphenyl)propanamide (**4b**). – Reactant: 1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-(3-benzylphenyl)propan-1-one (benzotriazolidine **3b**) (0.171 g, 0.5 mmol). The crude product was purified by column chromatography (eluent: cyclohexane/ethyl acetate/methanol 3:1:0.5).

N-(4-(6-methoxyquinolin-8-ylamino)pentyl)-2-(3-phenoxyphenyl)propanamide (**4c**). – Reactant: 1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-(3-phenoxyphenyl)propan-1-one (benzotriazolidine **3c**) (0.174 g, 0.5 mmol). The crude product was purified by column chromatography (eluent: cyclohexane/ethyl acetate/methanol 3:1:0.5).

N-(4-(6-methoxyquinolin-8-ylamino)pentyl)-2-(3-benzoylphenyl)propanamide (**4d**). – Reactant: 1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-(3-benzoylphenyl)propan-1-one (benzotriazolidine **3d**) (0.178 g, 0.5 mmol). The crude product was purified by column chromatography (eluent: cyclohexane/ethyl acetate/methanol 3:1:0.5).

N-(4-(6-methoxyquinolin-8-ylamino)pentyl)-2-(3-(hydroxy(phenyl)methyl)-phenyl)propanamide (**4e**). – Reactant: 1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-(3-(hydroxy(phenyl)methyl)phenyl)propan-1-one (benzotriazolidine **3e**) (0.179 g, 0.5 mmol). The crude product was purified by column chromatography (eluent: dichloromethane→dichloromethane/methanol 99.5:0.5→dichloromethane/methanol 99:1).

2-(2-(2,6-Dichlorophenylamino)phenyl)-N-(4-(6-methoxyquinolin-8-ylamino)pentyl)acetamide (**4f**). – Reactant: 2-(2-(2,6-dichlorophenylamino)phenyl)-1-(1H-benzo[d][1,2,3]triazol-1-yl)ethanone (benzotriazolidine **3f**) (0.199 g, 0.5 mmol). The crude product was purified by column chromatography (eluent: cyclohexane/ethyl acetate/methanol 3:1:0.5) and triturated with ether.

1-(4-Chlorobenzoyl)-2-methyl-5-methoxy-N-(4-(6-methoxyquinolin-8-ylamino)pentyl)-1H-indol-3-acetamide (**4g**). – Reactants: 1-(4-chlorobenzoyl)-2-methyl-5-methoxy-1H-indol-3-(ethan-2-one-2-(1H-benzo[d][1,2,3]triazol-1-yl)) (benzotriazolide **3g**) (0.229 g, 0.5 mmol), triethylamine (0.091 mL, 0.65 mmol). The crude product was purified by column chromatography (eluent: cyclohexane/ethyl acetate/methanol 3:1:0.5) and triturated with ether.

(3-(1-(4-(6-Methoxyquinolin-8-ylamino)pentylcarbamoyl)ethyl)phenyl)-(phenyl)methyl 4-(6-methoxyquinolin-8-ylamino)pentylcarbamate (**4h**). – Reactant: (3-(1-(1H-benzo[d][1,2,3]triazol-1-yl)-1-oxopropan-2-yl)phenyl)(phenyl)methyl 1H-benzo[d][1,2,3]triazole-1-carboxylate (benzotriazolide **3h**) (0.251 g, 0.5 mmol). The crude product was purified by column chromatography (eluent: dichloromethane/methanol 9:1) and triturated with acetone/petroleum ether.

DPPH radical-scavenging activity

Free radical scavenging activity (RSA) was evaluated by the scavenging of α,α -diphenyl- β -picryl hydrazyl (DPPH) radicals according to the method of Yen and Chen (13). Ethanolic solution of DPPH ($V = 1.0$ mL, $c = 0.16$ mmol L⁻¹) was added to 1.0 mL of either ethanolic solution of the test sample ($\gamma = 0.1$ – 0.5 g L⁻¹) or ethanol (negative control). The mixture was vortexed for 1 min and then left to stand at room temperature in the dark. After 30 min absorbance was read at 517 nm. Bleached DPPH solution, prepared by adding 1.0 mL of 0.16 mmol L⁻¹ DPPH solution to 1.0 mL of butylated hydroxyanisol (BHA) solution ($\gamma = 1$ g L⁻¹) was used as a positive control. RSA was calculated using $A_{\text{cont.}}$ (absorbance of the ethanol control) and A_{sample} (absorbance of the sample). RSA was expressed as the concentration that scavenges 50 % of DPPH free radicals (EC_{50}).

β -Carotene-linoleic acid assay

The antioxidant activity (ANT) of the selected twin drugs was evaluated using the β -carotene-linoleic acid system according to modified literature procedures (14). Tween 40 (200 mg) and 1.0 mL of β -carotene solution in chloroform ($\gamma = 0.2$ mg L⁻¹) were mixed. After removing chloroform in a rotary evaporator, 20 mg of linoleic acid and 30 mL of aerated distilled water was added to the oily residue with vigorous stirring. Aliquots (200 μ L) of thus obtained emulsion were added to 50 μ g of the test conjugate dissolved in 50 μ L of methanol (final concentration of the test compound 0.2 g L⁻¹). A reaction mixture containing 50 μ L of methanol instead of sample solution served as a control. BHA was used as an antioxidant standard. After adding the emulsion to the tubes, the plate was incubated at 50 °C for 2 h. During that period, the absorbance was measured at 450 nm at 15-minute intervals, starting immediately after sample preparation ($t = 0$ min) until the end of the experiment ($t = 120$ min).

The percent of antioxidant activity was calculated as described by Al-Saikhan *et al.* (15) using $R_{\text{cont.}}$ and R_{sample} , average bleaching rates of the water control and antioxidant (test compound or BHA), respectively. In addition, antioxidant activity was calculated from the absolute changes in absorbance at $t = 60$ and 120 min (AA-60 and AA-120,

respectively) (14). The results were normalized using two controls: a negative control with no protection (water) and a positive control with maximum protection (BHA). Accordingly, the antioxidant activity of the test compounds was expressed as:

$$AA = \left(1 - \frac{A_E^{t=0} - A_E^{t=t}}{(A_W^{t=0} - A_W^{t=t}) + (A_{BHA}^{t=0} - A_{BHA}^{t=t})} \right) \times 100$$

where $A_E^{t=0}$ is the absorbance of the conjugate at 0 min, $A_E^{t=t}$ is the absorbance of the conjugate at $t = 60$ or 120 min, $A_W^{t=0}$ is the absorbance of the water control at 0 min, $A_W^{t=t}$ is the absorbance of the water control at $t = 60$ or 120 min, $A_{BHA}^{t=0}$ is the absorbance of BHA at 0 min and $A_{BHA}^{t=t}$ is the absorbance of the BHA sample at $t = 60$ or 120 min.

Fe²⁺ chelating activity

The chelating activity (*ChA*) of PQ-NSAID conjugates toward ferrous ions was studied as described by Decker and Welch with some modification (16). To an aliquot of the methanolic solution of the test conjugate ($V = 150 \mu\text{L}$, $\gamma = 0.1\text{--}0.6 \text{ g L}^{-1}$), $50 \mu\text{L}$ of FeCl₂ solution ($c = 0.25 \text{ mmol L}^{-1}$) was added. After 5 minutes, the reaction was initiated by adding $100 \mu\text{L}$ of 1.0 mmol L^{-1} ferrozine solution. Absorbance at 545 nm was recorded after 10 min of incubation at room temperature. A reaction mixture containing $150 \mu\text{L}$ of methanol instead of conjugate solution served as a control. Quercetin was used as the chelating standard. *ChA* was calculated using A_{cont} (absorbance of the negative control, *e.g.*, blank solution without test compound) and A_{sample} (absorbance of the conjugate solution). Chelating activity was expressed as $ChEC_{50}$, the concentration that chelates 50 % of Fe²⁺ ions.

Statistical analysis

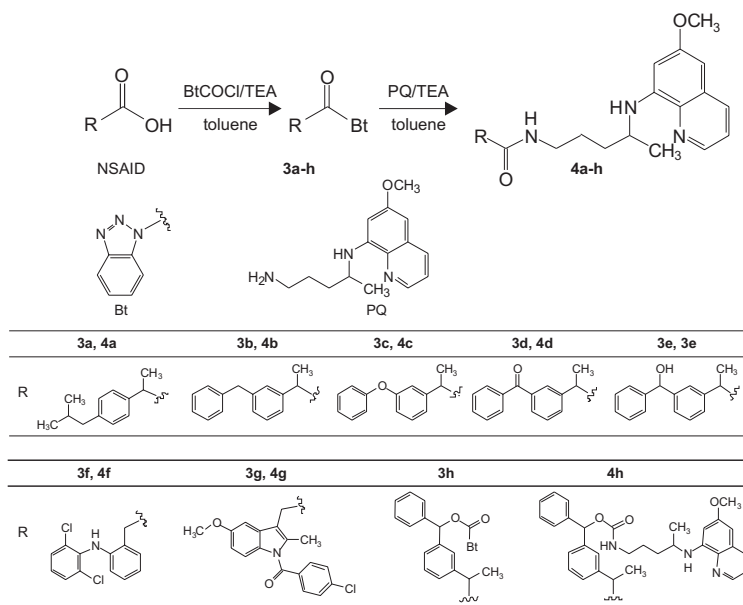
All assays described in the paper were performed in triplicate. The results were expressed as mean \pm SD. Statistical comparisons were made using Student's *t*-test or one-way ANOVA, followed by Dunnett's post-hoc test for multiple comparisons with the control. Statistical analyses were performed using the JMP V6 from SAS software (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Chemistry

The synthetic procedure leading to PQ-NSAID conjugates **4a–h** is presented in the Scheme 1. The first synthetic step involves preparation of NSAID benzotriazolides **3a–h** from the corresponding NSAID (ibuprofen, ketoprofen, fenoprofen, ketoprofen hydroxy and methylene analogues, diclofenac or indomethacin) and benzotriazole carboxylic acid chloride (BtCOCl, **1**). Benzotriazolides **3a–h** readily reacted with the primaquine

base. In that reaction, the primaquine terminal amino group formed an amide bond with the carboxylic group present in the title NSAIDs. The reaction was performed in toluene, at room temperature for 0.5 h. In general, 3-fold excess of TEA was used. Triethylamine formed a water soluble salt with benzotriazole, a by-product of the reaction, which was readily extracted with water. Synthesis of the PQ-indomethacin derivative **4g** was performed with benzotriazolidine **3g** : TEA molar ratio 1 : 1.3 to avoid indomethacin decomposition. Compound **4h** consists of one reduced ketoprofen moiety and two primaquine units (one bound by an amide and the other by the carbamate bond), and its preparation required a double amount of primaquine, *e.g.*, PQ to benzotriazolidine **3h** molar ratio 2 : 1.



Scheme 1

Structures of compounds **4a-h** were deduced from the analysis of their IR, ¹H and ¹³C NMR spectra and confirmed by elemental analysis. The chemical shifts were consistent with the proposed structures of the novel compounds. In ¹H NMR spectrum of amidocarbamate derivative **4h** each atom of two primaquine residues always appeared as one signal with a double integral, except for NH group at position 2: carbamate NH (2'') appeared at δ 7.47–7.39 ppm, while amide NH (2) had a signal at δ 7.95–7.91 ppm. Physicochemical and spectroscopic data are presented in Tables I and II.

Radical scavenging, antioxidant activity and Fe²⁺ chelating ability

The investigated conjugates demonstrated moderate antiradical activities, with EC₅₀ between 269.5 ± 10.7 and 379.3 ± 59.1 mg L⁻¹, with the exception of conjugate **4e**

Table I. Physicochemical and IR spectroscopic data for PQ-NSAID conjugates **4a–h**

Compd.	Yield (%)	M.p. (°C)	IR (ν_{\max} , KBr or NaCl, cm^{-1})	Molecular formula (M_r)	Elemental analysis calcd./found		
					C	H	N
4a	98	oil	3384, 3302, 3052, 2957, 2930, 1868, 1645, 1616, 1577, 1520, 1456, 1387, 1220, 1203, 1166, 1052, 822, 791	$\text{C}_{28}\text{H}_{37}\text{N}_3\text{O}_2$ (447.61)	75.13 75.49	8.33 8.40	9.39 9.20
4b	98	oil	3386, 3301, 3060, 3027, 2965, 2930, 1647, 1616, 1577, 1520, 1455, 1388, 1220, 1202, 1167, 1159, 822, 791	$\text{C}_{31}\text{H}_{35}\text{N}_3\text{O}_2$ (481.63)	77.31 77.48	7.32 7.44	8.72 8.60
4c	97	oil	3385, 3300, 3069, 2964, 2931, 1646, 1616, 1581, 1520, 1488, 1456, 1388, 1245, 1222, 1206, 1161, 1051, 932, 821, 791, 693	$\text{C}_{30}\text{H}_{33}\text{N}_3\text{O}_3$ (483.60)	74.51 74.39	6.88 6.71	8.69 8.95
4d	95	34–38	3380, 3310, 3059, 2965, 2932, 1655, 1616, 1596, 1578, 1520, 1455, 1388, 1284, 1221, 1202, 1159, 1052, 822, 791, 722, 705	$\text{C}_{31}\text{H}_{33}\text{N}_3\text{O}_3$ (495.61)	75.13 75.40	6.71 6.58	8.48 8.66
4e	85	oil	3391, 3310, 3061, 2965, 1932, 2870, 1650, 1616, 1578, 1520, 1454, 1388, 1221, 1202, 1159, 823, 791, 703	$\text{C}_{31}\text{H}_{35}\text{N}_3\text{O}_3$ (497.63)	74.82 74.98	7.09 7.39	8.44 8.22
4f	90	128–130	3393, 3298, 3070, 2964, 2941, 1661, 1633, 1616, 1591, 1578, 1521, 1454, 1389, 1205, 1158, 820, 768, 752	$\text{C}_{29}\text{H}_{30}\text{Cl}_2\text{N}_4\text{O}_2$ (536.17)	64.80 64.46	5.63 5.68	10.42 10.07
4g	77	113–116	3383, 3302, 3083, 2960, 2929, 1669, 1641, 1617, 1596, 1520, 1478, 1458, 1388, 1364, 1332, 1222, 1154, 1090, 823, 792, 754	$\text{C}_{34}\text{H}_{35}\text{ClN}_4\text{O}_4$ (599.12)	68.16 67.83	5.89 5.70	9.35 9.20
4h	93	82–86	3384, 3055, 2962, 2932, 1716, 1660, 1616, 1588, 1520, 1455, 1424, 1388, 1238, 1221, 1203, 1159, 1051, 1031, 822, 792	$\text{C}_{47}\text{H}_{54}\text{N}_6\text{O}_5$ (782.97)	72.10 72.44	6.95 7.09	10.73 10.35

Fig. 1. DPPH radical scavenging activities of the PQ-NSAID conjugates and butylated hydroxyanisol (BHA). Mean \pm SD, $n = 3$.

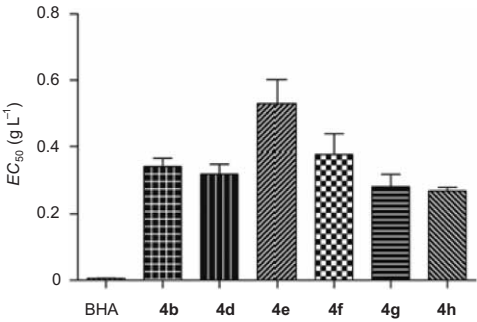
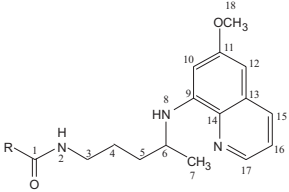
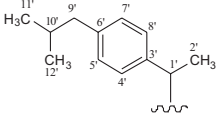
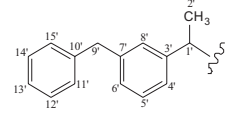
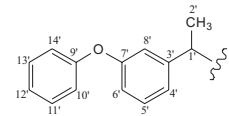
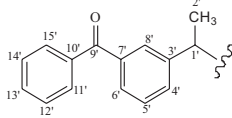
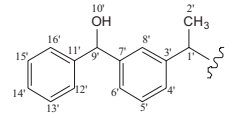
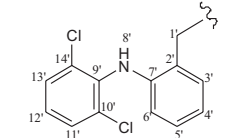
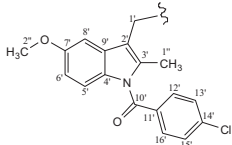
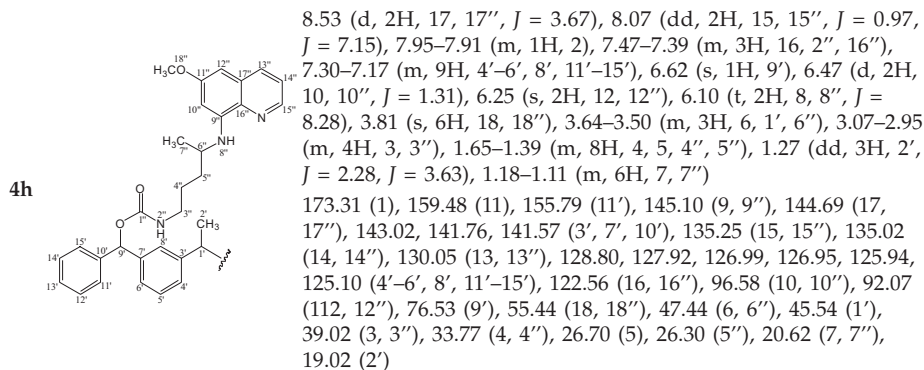


Table II. ¹H and ¹³C NMR data for PQ-NSAID conjugates 4a-h

			
Compd.	R	¹ H NMR (DMSO-d ₆ , δ/ppm, J/Hz)	¹³ C NMR (DMSO-d ₆ , δ/ppm)
4a		8.53 (d, 1H, 17, J = 3.79), 8.07 (dd, 1H, 15, J = 1.61, J = 6.65), 7.92–7.87 (m, 1H, 2), 7.42 (dd, 1H, 16, J = 3.96, J = 4.30), 7.19 (dd, 2H, 4', 8', J = 1.81, J = 6.25), 7.02 (dd, 2H, 5', 7', J = 2.02, J = 6.25), 6.47 (d, 1H, 10, J = 2.42), 6.25 (d, 1H, 12, J = 1.61), 6.07 (dd, 1H, 8, J = 2.42, J = 6.25), 3.82 (s, 3H, 18), 3.60–3.48 (m, 2H, 6, 1'), 2.96–2.87 (m, 2H, 3), 2.35 (d, 2H, 9', J = 6.65), 1.82–1.69 (m, 1H, 10'), 1.60–1.38 (m, 4H, 4, 5), 1.28 (dd, 3H, 2', J = 3.02, J = 3.83), 1.14 (dd, 3H, 7, J = 2.22, J = 3.93), 0.82 (d, 6H, 11', 12', J = 6.45)	173.68 (1), 159.49 (11), 145.09 (9), 144.67 (17), 140.15, 139.55 (3', 6'), 135.25 (15), 135.01 (14), 130.05 (13), 129.15, 127.34 (4', 8', 5', 7'), 122.55 (16), 96.55 (10), 92.06 (12), 55.44 (18), 47.43 (6), 45.26 (1'), 44.69 (9'), 38.91 (3), 33.77 (5), 30.06 (10'), 26.36 (4), 22.61 (11', 12'), 20.61 (7), 18.84 (2')
4b		8.53 (dd, 1H, 17, J = 1.55, J = 2.62), 8.08 (dd, 1H, 15, J = 1.54, J = 6.73), 7.91 (t, 1H, 2, J = 5.32), 7.44–7.40 (m, 1H, 16), 7.28–7.01 (m, 9H, 4'–6', 8', 11'–15'), 6.48 (d, 1H, 10, J = 2.53), 6.25 (s, 1H, 12), 6.09 (dd, 1H, 8, J = 1.61, J = 7.01), 3.88 (s, 2H, 9'), 3.82 (s, 3H, 18), 3.45–3.34 (m, 2H, 6, 1'), 2.95–2.87 (m, 2H, 3), 1.61–1.40 (m, 4H, 4, 5), 1.28 (dd, 3H, 2', J = 3.52), 1.14 (dd, 3H, 7, J = 1.55, J = 4.58)	173.47 (1), 159.49 (11), 145.09 (9), 144.69 (17), 143.03, 141.64, 141.43 (3', 7', 10'), 135.26 (15), 135.01 (14), 130.05 (13), 129.09, 128.82, 128.69, 128.08, 127.26, 126.37, 125.30 (4'–6', 8', 11'–15'), 122.57 (16), 96.59 (10), 92.07 (12), 55.45 (18), 47.36 (6), 45.57 (1'), 41.63 (9'), 38.98 (3), 33.79 (4), 26.37 (5), 20.63 (7), 18.99 (2')
4c		8.53 (dd, 1H, J = 1.51, 17, J = 2.65), 8.08 (dd, 1H, 15, J = 1.51, J = 6.81), 7.96 (t, 1H, 2, J = 5.39), 7.44–6.82 (m, 10H, 16, 4'–6', 8', 10'–14'), 6.48 (d, 1H, 10, J = 2.44), 6.26 (s, 1H, 12), 6.09 (dd, 1H, 8, J = 1.75, J = 6.90), 3.82 (s, 3H, 18), 3.61–3.52 (m, 2H, 6, 1'), 3.08–3.00 (m, 2H, 3), 1.62–1.39 (m, 4H, 4, 5), 1.29 (dd, 3H, 2', J = 3.50), 1.15 (dd, 3H, 7, J = 1.06, J = 4.94)	173.18 (1), 159.48 (11), 157.05, 156.89 (3', 7', 9'), 145.09 (9), 144.70 (17), 135.26 (15), 135.01 (14), 130.05 (13), 122.56 (16), 130.44, 130.15, 123.79, 122.79, 118.96, 117.99, 116.99 (4'–6', 8', 10'–14'), 96.58 (10), 92.08 (12), 55.44 (18), 47.35 (6), 45.44 (1'), 39.01 (3), 33.84 (4), 26.37 (5), 20.63 (7), 18.85 (2')

- 4d**
- 
- 8.52 (d, 1H, 17, $J = 4.18$), 8.07 (dd, 1H, 15, $J = 1.53$, $J = 6.64$), 8.04 (t, 1H, 2, $J = 5.49$, 2), 7.72–7.46 (m, 9H, 4'–6', 8', 11'–15'), 7.41 (dd, 1H, 16, $J = 4.09$, $J = 4.18$), 6.47 (d, 1H, 10, $J = 2.17$), 6.24 (t, 1H, 12, $J = 2.63$), 6.08 (dd, 1H, 8, $J = 4.03$, $J = 4.61$), 3.82 (d, 3H, 18, $J = 2.02$), 3.68 (q, 1H, 1', $J = 7.03$), 3.61–3.54 (m, 1H, 6), 3.10–3.00 (m, 2H, 3), 1.62–1.41 (m, 4H, 4, 5), 1.34 (t, 3H, 2', $J = 6.78$), 1.14 (dd, 3H, 7, $J = 5.76$, $J = 6.20$)
- 196.16 (9'), 173.16 (1), 159.47 (11), 145.08 (9), 144.68 (17), 143.33, 137.51, 137.29 (3', 7', 10'), 135.25 (15), 135.00 (14, 13), 133.09, 132.04, 130.05, 128.99, 128.94, 128.50 (4'–6', 8', 11'–15'), 122.55 (16), 96.56 (10), 92.07 (12), 55.44 (18), 47.35 (6), 45.39 (1'), 38.94 (3), 33.84 (4), 26.31 (5), 20.63 (7), 18.87 (2')
- 4e**
- 
- 8.53 (d, 1H, 17, $J = 4.13$), 8.08 (dd, 1H, 15, $J = 1.45$, $J = 6.77$), 7.93–7.91 (m, 1H, 2), 7.42 (dd, 1H, 16, $J = 4.05$, $J = 4.20$), 7.35–7.13 (m, 9H, 4'–6', 8', 12'–16'), 6.48 (d, 1H, 10, $J = 2.42$), 6.26 (t, 1H, 12, $J = 2.20$), 6.09 (dd, 1H, 8, $J = 3.13$, $J = 5.44$), 5.85 (d, 1H, 10', $J = 3.91$), 5.65–5.64 (m, 1H, 9'), 3.82 (s, 3H, 18), 3.61–3.56 (m, 1H, 6), 3.53 (q, 1H, 1', $J = 7.16$), 3.08–2.95 (m, 2H, 3), 1.61–1.58, 1.52–1.41 (2m, 4H, 4, 5), 1.29–1.26 (m, 3H, 2'), 1.17–1.14 (m, 3H, 7)
- 173.47 (1), 159.49 (11), 146.12, 145.98, 142.66 (3', 7', 10'), 145.11 (9), 144.71 (17), 135.27 (15), 135.02 (14), 130.06 (13), 128.46, 128.30, 127.10, 126.69, 126.03, 125.60, 124.89 (4'–6', 8', 12'–16'), 122.57 (16), 96.60 (10), 92.11 (12), 74.82 (9'), 55.46 (18), 47.46 (6), 45.64 (1'), 39.03 (3), 33.83 (4), 26.33 (5), 20.64 (7), 19.06 (2')
- 4f**
- 
- 8.53 (dd, 1H, 17, $J = 1.56$, $J = 2.66$, 17), 8.41 (s, 1H, 8'), 8.36 (t, 1H, 2, $J = 5.42$), 8.07 (dd, 1H, 15, $J = 1.52$, $J = 6.79$), 7.50 (d, 2H, 11', 13', $J = 8.08$), 7.42 (dd, 1H, 16, $J = 4.14$), 7.20–7.12 (m, 2H, 3', 5'), 7.05–7.00 (m, 1H, 12'), 6.85–6.80 (m, 1H, 4'), 6.47 (d, 1H, 10, $J = 2.41$), 6.30–6.27 (m, 2H, 12, 6'), 6.12 (d, 1H, 8, $J = 8.80$), 3.82 (s, 3H, 18), 3.67–3.56 (m, 1H, 6), 3.56 (s, 2H, 1'), 3.15–3.07 (m, 2H, 3), 1.69–1.49 (m, 4H, 4, 5), 1.17 (d, 3H, 7, $J = 6.42$)
- 171.89 (1), 159.49 (11), 145.12 (9), 144.69 (17), 143.42, 137.66, 129.84, 126.05 (2', 7', 9', 10', 14'), 135.26 (15), 135.01 (14), 130.05 (13), 122.55 (16), 130.80, 129.63, 127.60, 125.41, 121.10, 116.40 (3'–6', 11'–13'), 96.59 (10), 92.08 (12), 55.45 (18), 47.42 (6), 40.07 (1'), 39.18 (3), 33.83 (4), 26.24 (5), 20.68 (7)
- 4g**
- 
- 8.52 (dd, 1H, 17, $J = 1.52$, $J = 2.64$), 8.07 (dd, 1H, 15, $J = 1.45$, $J = 6.86$), 8.04 (t, 1H, 2, $J = 5.59$), 7.65 (q, 4H, 12', 16', 13', 15', $J = 8.64$), 7.42 (dd, 1H, 16, $J = 4.03$, $J = 4.21$), 7.11 (d, 1H, 5', $J = 2.38$), 6.94 (d, 1H, 6', $J = 8.97$), 6.69 (dd, 1H, 8', $J = 2.44$, $J = 6.60$), 6.47 (d, 1H, 10, $J = 2.38$), 6.25 (d, 1H, 12, $J = 2.38$), 6.10 (d, 1H, 8, $J = 8.71$), 3.81 (s, 3H, 18), 3.72 (s, 3H, 2''), 3.65–3.56 (m, 1H, 6), 3.48 (s, 2H, 1'), 3.12–3.05 (m, 2H, 3), 2.21 (s, 3H, 1''), 1.66–1.46 (m, 4H, 4, 5), 1.16 (d, 3H, 7, $J = 6.20$)
- 169.66 (1), 168.29 (10'), 159.47 (11), 156.00 (7'), 145.09 (9), 144.69 (17), 137.98 (14'), 135.53 (3'), 135.26 (15), 135.00 (14), 134.74 (4'), 131.58 (12', 16'), 131.35 (11'), 130.75 (9'), 130.04 (13), 129.47 (13', 15'), 122.57 (16), 115.01 (5'), 114.92 (2'), 111.67 (6'), 102.34 (8'), 96.57 (10), 92.07 (12), 55.83 (2''), 55.44 (18), 47.46 (6), 39.14 (3), 33.93 (4), 31.70 (1'), 26.50 (5), 20.63 (7), 13.82 (1'')



with $EC_{50} = 528.8 \text{ mg L}^{-1}$. BHA, investigated under the same conditions, had the EC_{50} value of 5.9 mg L^{-1} . The results are presented in Fig. 1.

Reduction of the absorbance of the β -carotene-linoleate emulsion in the presence of PQ-NSAID conjugates is shown in Fig. 2. All the investigated derivatives significantly inhibited β -carotene bleaching in comparison with the control. According to Amarowicz *et al.* (17), the normalized antioxidant activity at 60 and 120 min of incubation (AA-60 and AA-120) probably reflects the antioxidant activity of the test compound more accurately than the ANT value (Table III). Differences among the PQ-NSAID conjugates were minor. The most active conjugate, according both to the ANT value ($69.4 \pm 0.9 \%$) or AA-60 ($58.4 \pm 3.1 \%$) and AA-120 ($59.3 \pm 1.8 \%$), was conjugate **4h** with two primaquine units. The activity of diclofenac derivative **4f** was the lowest; however, activities of both **4h** and **4f** were not statistically different from the activity of the other conjugates.

Small differences in antioxidant, as well as in antiradical activities, could indicate that the primaquine moiety, which all the investigated substances have in common, might be responsible for both activities.

Under the conditions applied in our experiments only ketoprofen derivatives **4d** and **4e** demonstrated chelating activities with $ChEC_{50}$ of 1074.7 ± 23.2 and $861.9 \pm 50.4 \text{ mg L}^{-1}$, respectively. $ChEC_{50}$ of standard quercetin was $537.6 \pm 13.5 \text{ mg L}^{-1}$. Other more

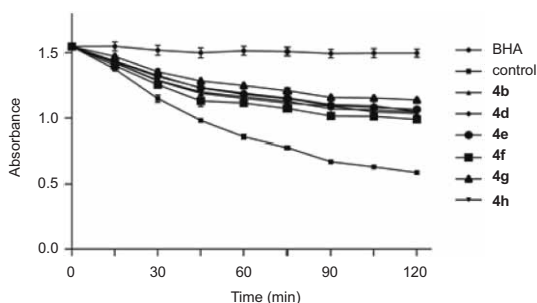


Fig. 2. Reduction of absorbance in the β -carotene-linoleate assay in the presence of the PQ-NSAID conjugates and butylated hydroxyanisol (BHA). Mean \pm SD, $n = 3$.

Table III. Antioxidant activity in the β -carotene-linoleate assay (ANT, AA-60, AA-120) and metal chelating activity (ChEC₅₀) of PQ-NSAID conjugates

Compd.	ANT (%) ^a	AA-60 (%) ^a	AA-120 (%) ^a	ChEC ₅₀ (mg L ⁻¹) ^b
4b	60.8 ± 1.8 ^{c,d,e}	44.8 ± 8.2 ^c	48.1 ± 5.2 ^{c,d}	ND
4d	63.2 ± 1.8 ^{c,f,g}	50.2 ± 8.9 ^c	50.5 ± 2.3 ^c	1074.7 ± 23.2 ^{c,d}
4e	62.8 ± 1.7 ^{c,h,i}	49.1 ± 5.9 ^c	52.0 ± 3.4 ^c	861.9 ± 50.4 ^{c,d}
4f	55.8 ± 1.7 ^{c,d,f,h,j,k}	39.8 ± 4.9 ^{c,d}	44.5 ± 3.1 ^{c,e}	ND
4g	61.4 ± 2.3 ^{c,j,l}	46.6 ± 6.3 ^c	50.1 ± 4.0 ^c	ND
4h	69.4 ± 0.9 ^{c,e,g,i,k,l}	58.4 ± 3.1 ^{c,d}	59.3 ± 1.8 ^{c,d,e}	ND
Standard ^{a,b}	96.6 ± 1.1	95.7 ± 5.2	95.0 ± 3.3	537.6 ± 13.5

ND - activity could not be determined due to precipitation.

ANT – Antioxidant activity relative to water control (determined at test compounds concentration of 0.2 g L⁻¹).

AA-60 and AA-120 – absolute changes in absorbance at $t = 60$ and 120 min (compounds concentration of 0.2 g L⁻¹).

Standards: ^a BHA (0.2 g L⁻¹), ^b quercetin.

Statistically significant difference ($p < 0.05$) within columns: ^c vs. standard, ^{d-l} between test compounds (the same letter indicates significant difference).

lipophilic derivatives precipitated upon addition of aqueous reagent solutions, so it was not possible to determine their chelating abilities.

Antiproliferative and antituberculotic activity

Antiproliferative activity of PQ-NSAID conjugates was screened *in vitro* on 4 human cell lines, which are derived from 4 cancer types. The following cell lines were used: HCT 116 (colon carcinoma), SW 620 (colon carcinoma), MCF-7 (breast carcinoma), and H 460 (lung carcinoma). Unfortunately, activities of all conjugates were too weak (concentration that causes 50 % growth inhibition was ≥ 100 $\mu\text{mol L}^{-1}$). Preliminary antituberculotic activity was checked as well. The minimal inhibitory concentration determined on the strain *Mycobacterium smegmatis* ATCC 14468 was too high (≥ 128 mg L^{-1}), so no further tests were done.

CONCLUSIONS

All the tested PQ-NSAID conjugates were found to possess moderate antiradical activity, with EC₅₀ between 269.5 ± 10.7 and 379.3 ± 59.1 mg L^{-1} , with the exception of conjugate **4e** with EC₅₀ = 528.8 mg L^{-1} . Conjugate **4h** exerted the strongest antioxidant activity, as determined by the β -carotene-linoleic acid assay (ANT = 69.4 ± 0.9 %; AA-60 and AA-120 were approximately 59 %). Moreover, primaquine derivatives with the keto-profen moiety demonstrated notable Fe²⁺ chelating ability. On the other hand, negligible antiproliferative and antituberculotic effects of conjugates **4a-h** were observed. Screening of antimalarial activity is in progress and the results will be published elsewhere.

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Acronyms. – AA-60 and AA-120 – absolute changes in absorbance at $t = 60$ and 120 min, respectively; ANT – antioxidant activity; BHA – butylated hydroxyanisole; Bt – benzotriazolyl; ChA – metal chelating activity; ChEC₅₀ – concentration that chelates 50 % of Fe²⁺ ions); DPPH – α,α -diphenyl- β -picryl hydrazyl; EC₅₀ – concentration that scavenges 50 % of DPPH free radicals; NSAID – non-steroidal anti-inflammatory drug; PQ – primaquine; ROS – reactive oxygen species; RSA – radical scavenging activity; TEA – triethylamine.

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S A Ž E T A K

Dvojni lijekovi primakina i nesteroidnih protuupalnih lijekova: Sinteza, hvatanje slobodnih radikala, antioksidativno djelovanje i keliranje Fe²⁺ iona

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U radu je opisana sinteza novih konjugata primakina s nesteroidnim protuupalnim lijekovima (PQ-NSAIDs, **4a-h**), njihova potpuna karakterizacija te testiranje sposobnosti hvatanja slobodnih radikala i antioksidativnog djelovanja. Sintetski postupak za pripremu dvojnih lijekova **4a-h** uključuje dva koraka: *i*) pripremu NSAID-benzotriazolida **3a-h** iz odgovarajućih nesteroidnih protuupalnih lijekova (ibuprofena, ketoprofena, fenoprofena, hidroksi i metilenskih analoga ketoprofena, diklofenaka i indometacina) i klorida 1-benzotriazol karboksilne kiseline (BtCOCl, **1**), *ii*) reakciju intermedijera **3a-h** s primakinom. Novi PQ-NSAID konjugati pokazuju umjerenu sposobnost hvatanja slobodnih radikala u DPPH testu te umjereno antioksidativno djelovanje u pokusu s β-karotenom i linoleinskom kiselinom. Osim toga, derivati ketoprofena **4d** i **4b** imaju primjetnu sposobnost keliranja Fe²⁺ iona. Svi konjugati **4a-h** pokazuju vrlo slabo antiproliferativno i antituberkulotsko djelovanje.

Ključne riječi: primakin, NSAID, dvojni lijek, konjugat, hvatanje slobodnih radikala, antioksidativno djelovanje, sposobnost keliranja

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